# CONSTRUCTION OF NOVEL STRAINS CONTAINING MINIMIZING GENOME BY Tn5-COUPLED Cre/loxP EXCISION SYSTEM

## 【Technical field】

The present invention relates to strains deleted specific chromosome sites, using transposon with loxP site and Cre/loxP site-specific recombination, and the construction method thereof. More particularly, the present invention relates to a method for constructing novel strains deleted specific chromosome sites, by Cre/loxP site-specific recombination using a transposon comprising a selectable marker and loxP site, and a Cre expression vector.

## 【Background Art】

Even though the technology to delete specific chromosome sites of E. coli by using Cre/loxP site specific recombination is generally known, it has been required to prepare targeting vector and perform PCR (polymerase chain reaction) in every experiment to delete specific chromosome sites in the conventional methods. The random transposition of transposon into the chromosome is also well known. However, it has not been reported that chromosome sites of microorganism can be deleted using transposon together with Cre/loxP site-specific recombination

## 【Detailed Description of the Invention】

The present invention relates to a method of deleting specific chromosome sites, using transposon and Cre/loxP site-specific recombination, in order to improve the conventional method requiring to prepare targeting vector and to perform PCR (polymerase chain reaction) in every experiment.

The present invention relates to a method of preparing strains deleted specific chromosome sites, using transposon and Cre/loxP site-specific recombination. More particularly the present invention relates to a method for developing novel strains deleted specific chromosome sites, by Cre/loxP site-specific recombination using transposon comprising a selectable marker and loxP site and Cre expression vector.

The method for developing novel strains deleted specific chromosome sites comprises the steps of:

(1) preparing two transposons comprising loxP site and different selectable markers;

(2) inserting the above two transposons into optional positions of microbial chromosome, respectively, and determining the inserted sites;

(3) integrating two transposons comprising different selectable markers into one chromosome by P1 phage transduction; and

(4) transforming a plasmid containing Cre gene into the host strain with the above chromosome and expressing it, to delete a chromosomal site between the two loxP sites of the transposons.

The preparation method can be explained in detail, as follows:

In the above step (1), the above two transposons have different selectable markers. In the Examples of the present invention, TnKGloxP and TnClox were prepared and used as the above two different transposons. TnKGloxP comprises loxP site (SEQ ID NO:4) and $Km^R$ (kanamycin resistant gene, SEQ ID NO:5) as a selectable marker and GFP(Green Flurolecent Protein, SEQ ID NO:6) gene. TnCloxP comprises loxP site (SEQ ID NO:4) and $Cm^R$ (chloramphenicol resistant gene, SEQ ID NO:7) as a selectable marker. The above two transposons have outer end transposase recognition sequences (OE sequence) comprising 19 base pairs at each terminus, which have SEQ ID NO:3 (5'-ctgtctcttatacacatct-3') and its reverse-complementary sequence (5'-agatgtgtataagagacag-3'), respectively.

In other words, transposon TnKGloxP has outer end transposase recognition sequences (OE sequence) comprising 19 base pairs at each terminus, loxP site, $Km^R$ as a selectable marker and GFP gene. Transposon TnCloxP has outer end transposase recognition sequences (OE sequence) comprising 19 base pairs at each terminus, loxP site and $Cm^R$ as a selectable marker. In the above transposons, the length and the sequence of the transposon can vary depending on the vector used for the preparation of transposon except the OE sequence at each terminus and loxP site, which are essential for recombination.

The above transposons, TnKGloxP and TnCloxP can also be obtained by PCR from the pTnKGloxP and pTnCloxP vectors,

respectively (see Figure 1).

According to one of the preferable examples, the above transposons TnKGloxP and TnCloxP may be prepared, as follow:

First, the preparation procedures of the above transposon TnKGloxP include the steps of:

- preparing a new vector pKGloxP by inserting GFP gene into pKKloxP vector comprising the linear $Km^R$ and loxP by using ligase;
- separating a DNA fragment comprising $Km^R$, GFP and loxP sites by treating pKGloxP vector with restriction enzyme;
- preparing pTnKGloxP vector by inserting the above separated DNA fragment into the linear pMODTM<MCS> vector by using ligase; and
- performing PCR of the above pTnKGloxp vector.

Also, the preparation procedures of the above transposon, TnCloxP include the steps of:

- separating a DNA fragment comprising $Cm^R$ and loxP sites by treating pKGloxP vector comprising $Cm^R$ and loxP sites with restriction enzyme;
- preparing pTnCloxP vector by inserting the above separated DNA fragment into the linear pMODTM<MCS> vector by using ligase; and
- performing PCR of the above pTnCloxP vector.

The following base sequences of the prepared Transposons TnKGloxP and TnCloxP according to the above methods, are shown as

SEQ ID NO:1and SEQ ID NO:2, respectively.

TnKGloxP base sequence

1 attcaggctg cgcaactgtt gggaagggcg atcggtgcgg gcctcttcgc tattacgcca

61 g***ctgtctctt atacacatct*** caaccatcat cgatgaattc gagctcggta cccgggttga

|| ← OE sequence→ ||

121 actgcggatc ttgcggcc**gc aaaaattaaa aatgaagttt tgacggtatc gaaccccaga**

|| ← $Km^R$

181 **gtcccgctca gaagaactcg tcaagaaggc gatagaaggc gatgcgctgc gaatcgggag**

241 **cggcgatacc gtaaagcacg aggaagcggt cagcccattc gccgccaagc tcttcagcaa**

301 **tatcacgggt agccaacgct atgtcctgat agcggtccgc cacacccagc cggccacagt**

361 **cgatgaatcc agaaaagcgg ccattttcca ccatgatatt cggcaagcag gcatcgccat**

421 **gggtcacgac gagatcctcg ccgtcgggca tccgcgcctt gagcctggcg aacagttcgg**

481 **ctggcgcgag cccctgatgc tcttcgtcca gatcatcctg atcgacaaga ccggcttcca**

541 **tccgagtacg tgctcgctcg atgcgatgtt tcgcttggtg gtcgaatggg caggtagccg**

601 **gatcaagcgt atgcagccgc cgcattgcat cagccatgat ggatactttc tcggcaggag**

661 **caaggtgaga tgacaggaga tcctgccccg gcacttcgcc caatagcagc cagtcccttc**

721 **ccgcttcagt gacaacgtcg agcacagctg cgcaaggaac gcccgtcgtg gccagccacg**

781 **atagccgcgc tgcctcgtct tggagttcat tcagggcacc ggacaggtcg gtcttgacaa**

841 **aaagaaccgg gcgcccctgc gctgacagcc ggaacacggc ggcatcagag cagccgattg**

901 **tctgttgtgc ccagtcatag ccgaatagcc tctccaccca agcggccgga gaacctgcgt**

961 **gcaatccatc ttgttcaatc atgcgaaacg atcctcatcc tgtctcttga tccactagat**

1021 **tattgaagca tttatcaggg ttattgtctc atgagcggat acatatttga atgtatttag**

1081 **aaaaataaac aaataggggt tccgcgcaca tttccccgaa aagtgccacc tgca**tcgatg

$Km^R$ → ||

1141 aattgatccg aagttcctat tctctagaaa gtataggaac ttcgaattgt cgacaagctt

1201 gatctggctt atcgaaatta atacgactca ctatagggag accggaattc **attatttgta**

|| ← GFP

1261 **gagctcatcc atgccatgtg taatcccagc agcagttaca aactcaagaa ggaccatgtg**

1321 **gtcacgcttt tcgttgggat ctttcgaaag ggcagattgt gtcgacaggt aatggttgtc**

1381 **tggtaaaagg acagggccat cgccaattgg agtattttgt tgataatggt ctgctagttg**

1441 **aacggatcca tcttcaatgt tgtggcgaat tttgaagtta gctttgattc cattcttttg**

1501 **tttgtctgcc gtgatgtata cattgtgtga gttatagttg tactcgagtt tgtgtccgag**

1561 **aatgtttcca tcttctttaa aatcaatacc ttttaactcg atacgattaa caagggtatc**

1621 **accttcaaac ttgacttcag cacgcgtctt gtagttcccg tcatctttga aagatatagt**

1681 **gcgttcctgt acataacctt cgggcatggc actcttgaaa aagtcatgcc gtttcatatg**

1741 **atccggataa cgggaaaagc attgaacacc ataagagaaa gtagtgacaa gtgttggcca**

1801 **tggaacaggt agttttccag tagtgcaaat aaatttaagg gtaagttttc cgtatgttgc**

1861 **atcaccttca ccctctccac tgacagaaaa tttgtgccca ttaacatcac catctaattc**

1921 **aacaagaatt gggacaactc cagtgaaaag ttcttctcct ttactcattt tttctaccgg**

1981 **tacccgggga tcctctagag tcgacctgca ggcatgcaag cttggcgtaa tcatggtcat**

2041 **agctgtttcc tgtgtgaaat tgttatccgc tcacaattcc acacaacata cgagccggaa**

2101 **gcataaagtg taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttgc**

2161 **gctcactgcc cgctttccag tcgggaaatc caagggc**gaa ttcgagctcg gtaccgggcc

GFP → ||

2221 ccccctcgag ggacct***ata acttcgtata gcatacatta tacgaagtta*** ***t***attaagggt

|| ← loxP site → ||

2281 tccggatcct ctagagtaga cctctagagt cgacctgcag gcatgcaagc ttcagggttg

2341 ***agatgtgtat aagagacag***c tgcattaatg aatcggccaa cgcgcgggga gaggcggttt

|| ← OE sequence → ||

2401 gcgtattggg cgctcttccg cttcctcgct cactgac

## TnCloxP base sequence

1 attcaggctg cgcaactgtt gggaagggcg atcggtgcgg gcctcttcgc tattacgcca

61 ***gctgtctctt atacacatct*** caaccatcat cgatgaattc gagctcggta cc**gcaaaaat**

|| ← OE sequence → || || ← $Cm^R$

121 **taaaaatgaa gttttaaatc aatctaaagt atatatgagt aaacttggtc tgacagttac**

181 **caatgcttaa tcagtgaggc accaataact gccttaaaaa aattacgccc cgccctgcca**

241 **ctcatcgcag tactgttgta attcattaag cattctgccg acatggaagc catcacagac**

301 **ggcatgatga acctgaatcg ccagcggcat cagcaccttg tcgccttgcg tataatattt**

361 **gcccatggtg aaaacggggg cgaagaagtt gtccatattg gccacgttta aatcaaaact**

421 **ggtgaaactc acccagggat tggctgagac gaaaaacata ttctcaataa acccttagg**

481 **gaaataggcc aggttttcac cgtaacacgc cacatcttgc gaatatatgt gtagaaactg**

541 **ccggaaatcg tcgtggtatt cactccagag cgatgaaaac gtttcagttt gctcatggaa**

601 **aacggtgtaa caagggtgaa cactatccca tatcaccagc tcaccgtctt tcattgccat**

661 **acggaatttc ggatgagcat tcatcaggcg ggcaagaatg tgaataaagg ccggataaaa**

721 **cttgtgctta tttttcttta cggtctttaa aaaggccgta atatccagct gaacggtctg**

781 **gttataggta cattgagcaa ctgactgaaa tgcctcaaaa tgttctttac gatgccattg**

841 **ggatatatca acggtggtat atccagtgat ttttttctcc attttagctt ccttagctcc**

901 **tgaaaatctc gataactcaa aaaatacgcc cggtagtgat cttatttcat tatggtgaaa**

961 **gttggaacct cttacgtgcc gatcaacgtc tcattttcgc caaaagttgg cccagggctt**

1021 **cccggtatca acagggacac caggatttat ttattctgcg aagtgatctt ccgtcacagg**

1081 **tatttattcg gcgcaaagtg cgtcgggtga tgctgccaac ttactgattt agtgtatgat**

1141 **ggtgtttttg aggtgctcca gtggcttctg tttctatcag** catcgatgaa ttgatccgaa

$Cm^R$ → ||

1201 gttcctattc tctagaaagt ataggaactt cgaattgtcg acaagcttga tctggcttat

1261 cgaaattaat acgactcact atagggagac cggaattcga gctcggtacc gggcccccc

1321 tcgagggacc ta***ataacttc gtatagcata cattatacga agttat***atta agatcctcta

|| ← loxP site → ||

1381 gagtcgacct gcaggcatgc aagcttcagg ***gttgagatgt gtataagaga cag***ctgcatt

|| ← OE sequences → ||

1441 aatgaatcgg ccaacgcgcg gggagaggcg gtttgcgtat tgggcgctct tccgcttcct

1501 cgctcactga c

As mentioned above, the base sequence and the length of the remainder parts, other than loxP site, OE sequence, $Km^R$ gene, GFP gene and $Cm^R$ gene sites, may vary depending on the vector used for

the preparation of transposon. The deletion, insertion, and/or substitution of one or more bases thereof does not change the function of transposon, if the base sequences of the loxP site, OE sequence, selectable marker ($Km^R$/GFP or $Cm^R$) are all included and preserved. Further, loxP site is located between the outer end transposase recognition sequences on both termini, and not inserted in the middle or the inside of the selectable marker, wherein it does not matter that loxP site is located in 3' side or 5' side of the selectable marker.

In the above step (2), transposases are added to the above two transposons TnKGloxP and TnCloxP, respectively, to form transposome, respectively; the transposomes including different transposons are transferred to different microorganisms using electrophoration; each transposon is inserted into the random site of the chromosomes of the microorganism; mutant microorganism inserted with the above transposon is selected; and the insertion site of the transposon in the selected mutants is identified. Since the random inserting function of the transposase can be activated by $Mg^{2+}$ ion, the formation of transposome is carried out in the absence of $Mg^{2+}$ ion, and the random insertion of transposon in the microorganism is carried out in the presence of $Mg^{2+}$ ion.

Also, since the two transposons include kanamycin resistant gene and chloramphenicol resistant gene, respectively, and thus, they are resistant against the above two antibiotics, the strains with the respective transposon can be selected by cultivating them in the kanamycin- or chloramphenicol-containing media after inserting the

above transposon thereto. The random insertion of transposon can be identified by Southern blot analysis, and the position of the inserted transposon can be confirmed by arbitrary PCR.

In the above step (3), a strain inserted with one transposon at one of the termini of the chromosomal site to be deleted and a strain inserted with other kind of transposon are collected from the strains in which the position of transposon have been identified. Using one of the selected strains as the donor and the other as recipient, the above two transposons are located on both ends of the chromosomal site to be deleted by general P1 phage transduction method, whrerein the loxP sites must be located in the same direction.

In the above step (4), Cre gene is transferred into the mutant strain containing transposons on both ends of the chromosomal site to be deleted by transferring the pTSCre expression vector into the above mutant strain. Since the transcription of the Cre gene in the pTSCre expression vector is controlled by tetracycline promoter (Ptet), Cre Recombinase is synthesize by expressing Cre gene by cultivating the mutant strain introduced with the above pTSCre expression vector in the medium containing chlorotetracycline. Cre Recombinase in this synthesis helps to remove the chromosomal site containing two loxP sites in either end by specifically cleaving the above two loxP sites and ligating the cleavage sites.

In addition, the preparation methods according to the present invention can additionally include the step of repeatedly performing steps (3) and (4) on the mutant strains containing deletions of a partial

chromosomal site to reduce the size of the chromosome by degrees. In other words, by randomly selecting two from the mutants containing deletions of the above specific chromosomal sites; fusing the chromosoms of the two selected mutants into one chromosome by P1 phage transduction to obtaining a new strain containing an extended chromosomal deletion site as large as the deleted sites of the above two mutants' chromosomes; and further extending the deleted site of the chromosome by continuously performing P1 phage transduction between the obtained new strain repeatedly and another mutant to reduce the size of the chromosome of mutant by degrees, a mutant with a larger deleted site of chromosome is obtained. In the continuous P1 phage transduction, in order to efficiently select the mutant with the desired deletion site, selectable marker acting as P1 recipient is removed by homologous recombination.

In the present invention, E. coli was used as the above microorganism, and Tn5 was used as the above transposon. It has been reported that Tn5 can be inserted into an random site of the chromosome (Berg, D. D., and M. M. Howe. 1989. Mobile DNA. American Society for Microbiology, Washington, D.C.), and Cre DNA recombinase recognizes the two loxP sites to catalyze the DNA recombination reaction between them (Abremski, K., HoessR., and Sternberg, N. 1984. Studies on the properties of P1 site-specific recombination. Cell 32, 1301-1311).

Also, P1 phage is known to have a function transferring a part of the chromosome of the host microorganism to other microorganism

(Watanabe, T., Furuse, C., and Sakaizumi, S. 1968. Transduction of various R factors by phage P1 in Escherichia coli and by phage P22 in Salmonella typhimurium). Therefore, the present invention develops a method of novel mutant microorganism containing deletion of a partial chromosomal site between two loxP sites, by inserting the transposon with loxP site into an random site of the microorganism chromosome, positioning two loxP sites inside a single chromosome in the same direction by using P1 phage transformation, and introducing Cre expressing vector to express Cre DNA Recombinase.

The invention will be further illustrated by the following examples. It will be apparent to those having conventional knowledge in the field that these examples are given only to explain the present invention more clearly, but the invention is not limited to the examples given.

## 【Brief Description of Drawings】

Figure 1 shows the preparation processes and the structures of transposons TnKGloxP and TnCloxP.

Figure 2 shows the steps of preparing E. coli in which a specific chromosomal site is deleted by using transposons TnKGloxP and TnCloxP.

Figur3 3A and 3B show the sites that can be inserted with TnKGloxP and TnCloxP in the E. coli genome.

Figure 4A through 4D show the method of deleting specific sites of E. coli chromosome by using transposons TnKGloxP and TnCloxP inserted in various sites and the PCR results of the deleted

chromosome.

EXAMPLE 1

**Preparation of transposons TnKGloxP and TnCloxP that can be inserted into random site in the E.coli chromosome**

Figure 1 represents a linear transposons TnKGloxP and TnCloxP, wherein TnKGloxP contains $Km^R$, GFP (pGFPuv, Clontech, Palo Alto, CA) and loxP, and TnCloxP contains $Cm^R$ and loxP sites. The above two transposons have outer end transposase recognition sequences (O Epicentre technologies, Madison, WI) comprising 19 base pairs at each terminus. As can be seen in Figure 1, the above transposons TnKGloxP and TnCloxP can be obtained by PCR from pTnKGloxP and pTnCloxP vectors, respectively.

The above pTnKGloxP vector was prepared by the following method. First, after GFP gene obtained from PCR was cleaved by EcoRI restriction enzyme (New England Biolabs, Beverly, MA), the GFP gene was inserted into vector pKKloxP (Michael D. Koob, et al, 1994, In vivo excision and amplification of large segment of the Escherichia coli genome, Nucleic Acids Research 22(12),2392-2398) which was cleaved by EcoRI restriction enzyme to be a linear form and with $Km^R$ and loxP sites, by using ligase (New England Biolabs, Beverly, MA), to obtain a novel vector. The novel vector was named as pKGloxP.

After separating the DNA fragment of 2.2 kb having $Km^R$ gene, GFP gene and loxP site by reacting the above pKGloxP with NotI/XbaI restriction enzyme (New England Biolabs, Beverly, MA), the separated

DNA fragment was inserted by using ligase into linear pMODTM<MCS> (Epicentre technologies, Madison, WI) treated with BamHI restriction enzyme, to obtain vector pTnKGloxP (see Figure 1).

The above pTnCloxP vector was prepared as follows: After separating 1.2 kb sized DNA fragment having $Cm^R$ and loxP sites by cleaving pKCloxP (Michael D. Koob, et al, 1994, In vivo excision and amplification of large segment of the Escherichia coli genome, Nucleic Acids Research 22(12), 2392-2398) vector containing $Cm^R$ and loxP sites with NotI and BamHI restriction enzymes (New England Biolabs, Beverly, MA), the above DNA fragment was inserted by using ligase into linear pMODTM<MCS> vector treated with BamHI restriction enzyme to prepare pTnCloxP vector (Figure 1).

Transposon TnKGloxP and TnCloxP were prepared from the above pTnKGloxP and pTnCloxP vectors, respectively by PCR. The primers used in the above PCR are pMOD<MCS>FP-1(SEQ ID NO:8) and pMOD<MCS>RP-1(SEQ ID NO:9) with the following base sequence.

pMOD<MCS>FP-1 : 5¢ATTCAGGCTGCGCAACTGT-3¢

pMOD<MCS>RP-1 : 5¢-TCAGTGAGCGAGGAAGCGGAAG-3¢

EXAMPLE 2

**Preparation of two kinds of *E. coli* mutant libraries inserted**

**with transposons TnKGloxP and TnCloxP into ranom sites of *E. coli* chromosome and identification of the insertion site**

After reacting 500 ng of TnKGloxP and 500 ng of TnCloxP with 10 μl of Tn5 transposase, respectively, and adding DDW (Double Distilled Water) to make the total volume of the solution to 20 μl, each transposome was formed by reacting at 25 °C for 30 min. To inhibit the random insertion function of the transposase, the above reaction was carried out in the absence of $Mg^{2+}$ ion. One microliter of the transposome was transferred into E. coli strain MG1655 (Seoul National University Biological Sceince Division, Dr. J. H. Noh) by conventional electrophoration method [Bio-RAD, Bacterial electro-transformation and Plus Controller Instruction Manual, Cat.No 165-2098 ; Thompson, JR, et al. An improved protocol for the preparation of yeast cells for transformation by electrophoration. Yeast 14, 565-571 (1998) ; Grant, SG, et al. Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methyllation-restriction mutants. Proc. Natl. Acad. Sci. USA 87, 4645-4649 (1990)], in the presence of $Mg^{2+}$ ion. LB medium (tryptone 1%, yeast extract 0.5%, NaCl 0.5%) was used for the cultivation of the strain, wherein a small amount of $Mg^{2+}$ ion was contained.

Therefore, the random insertion function of transposase can be activated by $Mg^{2+}$ ion inside E.coli cells, and transposome can be inserted into the E. coli chromosome at random sites. Since the E.coli mutant comprising the above two transposons inserted have kanamycin or chloramphenicol resistance due to $Km^R$ or $Cm^R$ gene in the

transposons, they were selected in the kanamycin or chloramphenicol media. Each transposon inserted into the chromosome of E.coli MG1655 was identified by Southern blot analysis.

The conditions for Southern blot analysis were as follows: After separating the chromosomal DNA from the E. coli mutant in which transposon was inserted and cleaving the chromosomal DNA by using ClaI restriction enzyme (New England Biolabs, Beverly, MA), electrophoresis was performed on 1 % agarose gel. DNA in the agarose gel was transferred onto Hybond N+ membrane (Amersham) and the transferred DNA was blotted by using $^{32}$P-labled $Km^R$ or $Cm^R$ gene as probe. The transposon insertion site was confirmed by arbitrary PCR (Caetano-Annoles, G. 1993. Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Appl., 3, 85-92.).

DNA around the transposon insertion site was amplified using Tn5 insertion sequence specific primer and the primer that can arbitrarily bind to the chromosomal DNA at the outer side of transposon. The above arbitrary PCR was composed of two steps:

In the first step, single strand DNA containing the end sequence of transposon and the outer sequence thereof by using transposon specific primer Tn5Ext (5¢AGCATACATTATACGAAGTTATATTAAG-3¢, synthesized by Genotech), and subsequently, the primer Arb1 (5¢TTGAGCGATAGACGTACGATNNNNNNNNNNGATAT-3¢, synthesized by Genotech) binding to nonspecific site was bound to an nonspecific site of the above synthesized single strand DNA, to

synthesize double strand DNA.

In the second step, the above synthesized double strand DNA was amplified in large scale by using transposon specific primer Tn5Int and primer Arb2 (5¢TTGAGCGATAGACGTACGAT-3¢, synthesized by Genotech) whose base sequence is identical to 25 sequence of 3' end of Arb1. The amplified DNA was separated from the agarose gel by using Qiaquic spin PCR purification kit (Quiagen), the base sequence of the above separated DNA was analyzed using primer Tn5Int (5¢ TCGACCTGCAGGCATGCAAGCTTCA-3¢, synthesized by Genotech), and the insertion site was identified by comparing the above analysis result with Gene Bank DNA sequence by using BLAST program. The identified insertion sites of TnKGloxP and TnCloxP by the above methods are shown in Figure 3.

EXAMPLE 3

**Construction of *E. coli* mutant containing two transposons on the chromosome by P1 phage transduction**

Two mutants with the two different transposons at one end and the other end of the specific chromosomal site to be removed, respectively, were selected from the TnKGloxP mutant library and TnCloxP mutant library prepared according to Example2. Then, the two chromosomes with the different transposons were integrated in a single chromosome by P1 phage transduction so that one transposons should be located on one end of the chromosomal site to be removed

and the other transposon should be located on the other end. The loxP was positioned in the same direction, and P1 phage transduction was carried out by following a well-known protocol (Miller, J., H., editors, 1992, A short Course in Bacterial Genetics; A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, New York: Cold Spring Harbor).

EXAMPLE 4

**Construction of *E. coli* mutant containing deletion mutation by expressing Cre DNA recombinase by transferring pTSCre expression vector**

pTSCre expression vector (Yoon YG, et al, 1998, Cre/loxP-mediated excision and amplification of the Eshcerichia coli genome, Gene 14, 89-95) was transferred into the E.coli mutant with TnKGloxP and TnCloxP on either end of the chromosomal site to be deleted. Since the transcription of the Cre gene existing in the pTSCre expression vector is controlled by tetracycline promoter (Ptet), the mutant was cultivated in the medium containing chlorotetracycline at 42 °C, to express Cre recombinase. E.coli mutant with chromosome deletion mutation obtained from the result of expression of Cre DNA recombinase was confirmed by PCR.

In the Examples 3 and 4, a mutant in which TnKGloxP was inserted at b0532 site and a mutant in which TnCloxP was inserted at b0619 site; a mutant in which TnKGloxP was inserted at b2011 site and a mutant in which TnCloxP was inserted at b2073 site; a mutant in

which TnKGloxP was inserted b2829 site and a mutant in which TnCloxP was inserted at b2890 site; and a mutant in which TnKGloxP was inserted at b4271 site and a mutant in which TnCloxP was inserted at b4326 site were used for P1 transduction to express Cre gene. The results are shown in Figures 4A, B, C and D.

EXAMPLE 5

**Construction of mutant with extended chromosomal deletion site by repeatedly performing P1 phage transduction method.**

The chromosomal deletion site of E. coli mutant containing a specific chromosomal sites were deleted was extended by P1 phage transduction as mentioned in the above Example 3 and 4. First, two mutants were selected from the above specific chromosome site deleted mutants. Using one of them as the donor, and the other as recipient of P1 phage lysate, a new mutant was prepared, in which all of the chromosomal deletion sites of the above two mutants were deleted. Then, this mutant was used again as P1 phage recipient, and the already prepare mutant containing other chromosomal deletion site was used as donor to perform P1 phage transduction continuously and repeatedly. By this method, a chromosomal deletion site of other mutant was repeatedly removed from the chromosome of the above obtained mutant. In the continuous P1 phage transduction, in order to efficiently select the microorganism, selectable marker that acts as P1 recipient was eliminated by homologous recombination.

【Industrial Applicability】

As mentioned above, the present invention related to a method of developing novel *E. coli* strain deleted specific chromosome sites, using transposon and Cre/loxP site-specific recombination. Various sites of *E. coli* chromosome can be selectively and efficiently removed, and library of *E. coli* mutants containing deletions of specific chromosomal sites can be obtained, and a variety of E. coli mutants with selectively reduced chromosome can be created by repeatedly performing P1 phage transformation and reducing the chromosome by degrees. By the method of the present invention, nonessential gene for the growth of *E. coli* can be removed, and genetically simplified *E. coli* mutant can be constructed that can be used for the functional research in genomics. Also, fast growing *E. coli* mutant can be selected and used as artificial cell line, since nonessential genes for growth were eliminated. Also, the present invention can be applied to other microorganisms other than *E. coli*, and therefore a variety of mutant microorganisms can be created with selectively reduced chromosomes. Also, in the minimized chromosome prepared according to the method of the present invention, a cassette made by collecting foreign genes related to new metabolism can be constructed and introduced in organisms, to create novel organisms having a variety of useful functions